THE INTERACTION OF SPERMINE AND ETHYL METHANE SULFONATE ON SEX-LINKED LETHALS IN *DROSOPHILA MELANOGASTER*

A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

---

by

Richard Joseph LeRoy

August 1972

THE INTERACTION OF SPERMINE AND ETHYL METHANE SULFONATE ON SEX-LINKED LETHALS IN DROSOPHILA MELANOGASTER

by

Richard Joseph LeRoy

Approved by Committee:

Michael E. Myszewski
Chairman

Wayne B. Merkley

for D. Woods

Sale I Canfield
Dean of the School of Graduate Studies

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The structure of deoxyribonucleic acid (DNA) was determined by Francis Crick and James D. Watson. Since their discovery, the structure and action of DNA has been studied extensively. DNA has been proven to constitute the genetic material and is therefore responsible for initiating the production of proteins within the cell. The genes on the DNA molecule are arranged in a linear sequence and are composed of nucleotides. There are four different nucleotides in higher animals: (1) adenine, (2) thymine, (3) cytosine, and (4) guanine. The chemical properties of the bases in the nucleotides causes adenine to join with thymine and cytosine to join with guanine to form a double helical DNA molecule that resembles a ladder. The steps of the ladder are represented by two nucleotides such as cytosine-guanine or adenine-thymine. A gene is represented by a long sequence of nucleotides in a very definite arrangement. The precise arrangement of nucleotides will result in a precise message to the ribosomes of the cell for the production of a protein that will have a specific linear sequence of amino acids as directed by the sequence of nucleotides that make up the DNA molecule.

If the sequence of nucleotides is altered the message that is given to the ribosomes is also altered and a new protein could be produced. An alteration of the nucleotide

sequence would be called a mutation. If the alteration of the nucleotide sequence is the result of a certain applied chemical or physical phenomenon, then the change in the nucleotide sequence is said to be an induced mutation. Induced mutations can be caused by ionizing irradiation, mustard gas, urethane, phenols, formaldehyde, ethylmethane sulfonate (EMS) and others (Auerbach, 1967). Spontaneous mutations are those alterations in the nucleotide sequence resulting from unrecognizable stress.

Ethylmethane sulfonate (EMS) ($CH_3CH_2OSO_2CH_3$) is a monofunctional alkylating agent that has been widely accepted as a potent mutagenic agent for Drosophila. EMS affects primarily post-meiotic sperm cells which have completed DNA replication and apparently works on one strand of the DNA molecule (Khan, 1969).

EMS may be applied to Drosophila in several ways. In the feeding method, 0.15% EMS is mixed with 1.0% sucrose solution which is then used to saturate absorbent paper. Adult males are then allowed to feed for a 24 hour period on the sugar and EMS solution. It has been shown that this method of application will produce a frequency of sex-linked recessive lethal mutation 38.7% (Khan, 1969).

In no case has the storage of sperm in Drosophila treated with EMS and other monofunctional alkylating agents led to a change in delayed and complete germinal mutations. Apparently, monofunctional alkylation of the Drosophila

X-chromosome yields an effect which is not influenced further by allowing time for the completion of further reaction or loss of reactions (Khan, 1969).

The mechanism of this form of mutation (alkylation) is caused by depurination of DNA. Depurination of DNA can result when the single alkyl group on the monofunctional EMS molecule attacks an electron-rich site at the N-7 position of the purine. This will cause a weakening of the glycosidic bond between purine and ribose sugar causing a cleavage of the purine from the DNA. This gap in the DNA can be filled with any of the four bases so that a transversion or transition mutation can result (Bautz and Freeze, 1960).

A transition mutation results when a purine replaces another purine, that is adenine replaces guanine or vice versa. A transversion mutation occurs when a purine is replaced by a pyrimidine such as cytosine or thymine (Auerbach, 1967).

Johnson and Bach (1965) have concluded that spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$ behaves as an antimutagenic agent with bacteria and that the antimutagenic affect is more pronounced when bacteria are exposed to a mutagenic agent like caffine or ultraviolet light.

Johnson and Bach (1965) tested the antimutagenic abilities of spermine in a culture media of tryptophan-requiring mutants of Escherichia coli and Staphylococcus

aureus. After a short growing period on spermine-supplemented media, the cultures were inoculated in a new media containing streptomycin sulfate. The capability of spermine to act as an antimutagen was determined by counting new colonies of bacteria that occurred in the antibiotic culture media. In all cases cells grown in the presence of spermine had a significantly lower mutation rate than the control cells.

It is generally believed that the site of the antimutagenic action of spermine is the DNA molecule. The exact method by which spermine is attached to the DNA molecule is not known; however, it is generally accepted that it forms a bridge across the double helix with one end of the spermine molecule combined with the phosphate group and the other end possibly attached to the adenine or thymine group. It has been suggested that spermine may have all four of its amino groups interact to form ionic bonds with DNA (Stevens, 1967).

Investigations by Johnson and Bach (1968) have shown that most of the spermine that enters a cell combines with DNA, but t-RNA and ribosomes will also be able to react and combine with spermine. The affinity between t-RNA and spermine increases as the pH decreases. It is therefore expected that the amount of spermine attached to the DNA would decrease as the pH decreases. Also, it has been suggested that spermine, having a cationic nature, is sought

after by the various anionic species that are found within the cell.

Spermine causes an increase in the melting temperature of DNA and the exact extent of this increase has been shown to be a function of the adenine and thymine content of the DNA. However, research by Hirschman et al. indicates that there is little correlation between the binding of spermine and the base composition of DNA (Hirschman, Leng and Felsenfeld, 1967).

Dimethyl sulfoxide (DMSO) (C_2H_6SO) is an aprotic solvent with a number of unique solvation properties. It can cause the dissociation of acids and bases and affect the rates and mechanisms of reactions (Parker, 1962). This compound has been used as a biological preservative to prevent freezing and damage of living cells and has proven to be a radioprotective agent in mice (Ashwood-Smith, 1961).

Although DMSO may have long term side effects in humans which make it unsuitable for use as a routine drug, it has been proven to have a very low toxicity level to mice when applied to tissues in high concentrations (Dixon et al., 1965). Bovine and human spermatozoa can be suspended and frozen in DMSO without any apparent damage (Lovelock and Bishop, 1959).

There is considerable evidence that DMSO has the ability to carry large molecules across the cell membrane without damaging the cell membrane. When DMSO was applied

to chironomid salivary glands there was little observable effect, except that the chromosomes appeared slightly more diffuse than the normal salivary gland chromosome. However, when DNase (M.W. 40,000 to 60,000) was mixed with DMSO, the contents of the nucleus were no longer observable after twenty minutes (Berry and Dietz, 1968). The molecular weight of spermine is 184 and that of EMS is 124. If DMSO can carry DNase through the cell membrane without any apparent damage to the cell membrane, then DMSO should be able to carry low molecular weight substances such as spermine and EMS through the cell membrane without detrimental effects.

The mechanism by which DMSO is able to overcome permeability barriers is not well understood. It appears that the increased permeability is not caused by poisoning of active transport, but involves changes in the structure of the membrane. The movement of DMSO through the skin in some way influences the movement of other molecules through the skin (Franz and Van Bruggen, 1967).

Berry and Dietz (1968) reported that one consistent feature of all DMSO-treated cells was that although membrane-bounded organelles were considerably swollen, the membranes or membrane itself appeared to be unbroken as observed under the light and electron microscopes. They also observed that no change in the nuclear membrane could be seen except the nuclear pores became slightly enlarged.

When Drosophila were dipped into 0.1M DMSO solution

the sex-linked recessive lethal mutation rate as observed by Muller-5 tests was 0.0%. Flies that received DMSO by a dipping treatment and then exposed to 2000r X-ray had a slightly higher but insignificant sex-linked recessive lethal mutation rate than flies that received only X-ray treatment. A 4.4% dominant lethal mutation rate was observed for flies that were dipped into 0.1M DMSO (Alexander, 1966).

This study will attempt to determine if spermine can act as an antimutagenic agent in an organism other than bacteria, namely Drosophila. The topical application of DMSO-EMS solution to Drosophila should establish a mutation rate that can be compared with the mutation rate produced from the topical application of DMSO-EMS-spermine. If the mutation rate of the DMSO-EMS treated flies is significantly higher than the mutation rate of the DMSO-EMS-spermine treated flies, then spermine could be considered as an antimutagenic agent in higher and more complex organisms such as Drosophila. If the mutation rate of DMSO-EMS-spermine treated flies is not significantly different from the DMSO-EMS treated flies then the interaction of EMS and spermine prevented spermine from acting as an antimutagen.

MATERIALS AND METHODS

In the present experiment, EMS ($CH_3CH_2OSO_2CH_3$) was used to induce mutations in Drosophila melanogaster. Drosophila melanogaster is a fruit fly that has been widely

used in genetic studies. It was planned that EMS treated flies would produce a mutation rate significantly above the spontaneous mutation rate of the untreated flies. The two strains of Drosophila used for this experiment was a wild-type obtained from Michigan State University and a Muller-5 (Basc) strain from the University of Oregon.

In addition to treatment of EMS, some flies were treated with dimethyl sulfoxide (C_2H_6SO) and some flies received treatments of DMSO and spermine. DMSO is a chemical that has the ability to carry large molecules across the cell membrane. Spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$ has been recorded to be an antimutagen in bacteria (Johnson and Bach, 1965). These experiments test the hypotheses that spermine acts as an antimutagen in Drosophila. If spermine acts as an antimutagen in organisms more complex than bacteria, then flies that are treated with EMS, DMSO and spermine should have a lower mutation rate than flies treated with just the DMSO and the mutagen EMS.

The mutations that were detected were mutations that occurred on the X-chromosomes. The detection of these mutations was accomplished by applying the Muller-5 test. The Muller-5 test is an objective test that utilizes the Muller-5 (Basc) strain of Drosophila melanogaster. Basc X-chromosomes have complex inversions that act as cross-over suppressors when carried heterozygously with any wild-type X-chromosome. The Basc X-chromosomes carry markers that include an

incomplete dominant mutant gene for bar eyes and a recessive gene producing apricot colored eyes (Alderson, 1965).

The Muller-5 fly is phenotypically characterized by the presence of a narrow vertical bar-shaped eye that has about 90 facets in the male and about 70 facets in the female. The normal number of facets per eye is between 740 and 780. The eyes of the male are yellowish-orange while the female's are slightly lighter in color than the male (Lindsley and Grell, 1967).

The wild-type Drosophila used in these experiments was originally collected in Roseburg, Oregon, and have been named Oregon R. (OR) (Lindsley and Grell, 1967).

In this experiment, the OR males, which carry a single wild-type X-chromosome were treated and mated with homozygous Basc virgin females. Any lethal mutation observed in the F_2 generation will result from a recessive mutation that occurred on the treated wild-type X-chromosome. This kind of mutation is called a sex-linked recessive lethal mutation. Autosomal dominant mutations cannot be counted in adult flies because organisms with these mutations die in the egg or larva stages (Spencer and Stern, 1948; Alderson, 1965). Thus, the only mutations that were scored were those that occurred on the X-chromosome and were recessive and lethal in nature.

Wild-type Oregon-R (OR) males were treated in six different ways: (1) Control group, no treatment, (2) 1.0M DMSO applied topically, (3) 24 hour feeding of 0.025M EMS in

1.0% sucrose solution suspended on absorbent paper, (4) 1.0M DMSO mixed with 0.02M spermine applied topically, (5) 1.0M DMSO, 0.025M EMS and 0.02M spermine applied topically, and (6) 1.0M DMSO and 0.025M EMS applied topically. Test solutions were prepared so that the molarity of chemicals in the solutions remained the same.

A syringe microburet (model number SB2, from Micrometric Instruments Co., Cleveland, Ohio), was used with a SIYT syringe in 0.5 microleter divisions to place 0.5 microleters of test solution on the side of a glass vial. OR males were anesthesized and placed in this solution so that the posterior ventral portion of the abdomen would be in the center of the drop. The fly would remain anesthesized on the drop until all the test solution was dried. It would take ten to fifteen minutes for the chemical solution to dry. Most of the flies would then be able to walk, but some flies would get their wings stuck to the vials. Gentle tapping of the vial would eventually free any flies stuck to the vial. Any flies that did not show good vitality within an hour after treatment would not be used for mating. Flies that did show good vitality were mated within an hour after treatment to virgin Basc females.

All flies were reared in continuous light at 25°C growing in about three-fourths inch of media prepared from 1 liter water, 120 ml unsulfured molasses, 5g agar, 100g corn meal, 20g Brewers yeast and 4.5 ml of propionic acid.

Wild-type males were collected every eight hours for a twenty-four hour period. During the next twenty-four hour period the flies were taken to another laboratory building where they were treated. After treatment and recovery the flies were carried back to the mating and storage laboratory.

For the P_1 cross, a virgin female Basc was mated to a treated OR male. The treated male was put into a quarter pint size bottle with a female immediately after recovery for a twenty-four hour mating period. After the twenty-four hour mating period the males were removed and the Basc females were allowed to lay eggs for three more days.

From the P_1 cross of OR males and Basc females, all F_1 males carried a single Basc X-chromosome and a Y-chromosome. Phenotypically these F_1 males had bar-shaped and apricot color eyes. All F_1 females contained one paternal wild-type X-chromosome and one maternal Basc X-chromosome. The phenotype of the F_1 female was characterized by a red kidney-shaped eye. The complex inversions of the Basc X-chromosome are important at this point because the Basc chromosome suppresses crossing-over with the paternal wild-type X-chromosome. Thus the X-chromosome from the treated P_1 OR male is transmitted intact.

To produce the F_2 generation, the F_1 siblings were allowed to mate. From this cross two phenotypic classes of males and two phenotypic classes of females were produced in the cultures not carrying a lethal mutation. The female

progeny that were produced were: (1) homozygous Basc, which is distinguished by having an apricot-colored and bar-shaped eye, and (2) heterozygous females containing one wild-type X-chromosome and one Basc X-chromosome. The heterozygous class females can be recognized by having a red kidney-shaped eye. The two classes of males that were produced in the absence of a sex-linked recessive lethal mutation were: (1) those having the paternal, wild-type X-chromosome that resulted in normal red eyes, and (2) the class of male with one Basc X-chromosome exhibiting a bar-shaped and apricot-colored eye.

A sex-linked recessive lethal was scored by the absence of the wild-type male progeny class in the F_2 generation. An F_3 generation was used to verify any presumptive sex-linked recessive lethal mutation; this cross was between a heterozygous F_2 female and a Basc male. A cross to the F_3 generation was run on any F_2 culture producing six or less Basc males and no wild-type males. If only six or fewer Basc males were represented in the F_2 progeny, then the possibility becomes 1 in 64 that the eggs fertilized were the eggs that contained the untreated maternal X-chromosome.

An egg hatching test was run in conjunction with all chemicals tested in this experiment to evaluate the frequency of post-fertilization mortality. For these tests OR males were treated identically to the treatment given the OR males in the sex-linked recessive lethal test.

Following treatment and recovery, the wild-type males were placed in vials and were mated individually to Basc females for twenty-four hours. After the twenty-four hour period the males and females were removed from the first vials. The males were destroyed and each female was put individually into a second vial so that she could lay eggs for a second twenty-four hour period. The females were repeatedly transferred to new vials for several twenty-four hour periods.

The number of eggs that were layed in a vial were counted. After twenty-four hours the eggs were observed and the number of eggs that had not hatched were counted. By comparing the number of eggs that had not hatched with the total number of eggs, an estimate could be made of post-fertilization mortality.

The number of eggs that did not hatch in the control group could represent a background mutation rate for dominant lethal mutations. This background rate could then be used for comparison with any induced dominant lethal mutation that occurred with the different chemicals tested.

RESULTS

Sex-Linked Recessive Lethals

The sex-linked recessive lethal mutation rate for the control group was 0.27%. When the control data is compared to the DMSO data, which has a sex-linked recessive lethal

mutation rate of 0.0%, a 0.06 chi-square value is obtained (Table 1). Alexander (1966) also recorded a 0.0% sex-linked recessive lethal mutation rate for flies dipped in DMSO, and a 0.0% sex-linked recessive lethal mutation rate for the non-treated flies. The chi-square value obtained in this experiment indicated that there is no significant variation of mutation rates between DMSO treated flies and non-treated control flies. Apparently, there is no mutagenic effect of DMSO as applied in these experiments.

When the control group is compared to the DMSO-spermine treated flies, which have a 0.28% sex-linked recessive lethal mutation rate, a 0.35 chi-square value is obtained (Table 1). This chi-square value again indicates that flies treated with DMSO and spermine do not have a mutation rate significantly different from the control group.

In a comparison between the control group and the flies treated with DMSO-EMS, which have a 2.5% sex-linked recessive lethal mutation rate, a 4.95 chi-square value is obtained (Table 1). This chi-square value indicates a significant difference in mutation rate as compared to the control group. Apparently, the EMS mutagen is being carried across the exoskeleton, abdominal membranes and various tissues, inducing mutations on the chromosomes of the sperm.

A comparison between the DMSO-spermine treated group which has a 0.28% sex-linked recessive lethal mutation rate, and the DMSO-EMS-spermine treated flies which have a 2.45%

Table 1. Sex-linked recessive lethal mutation results.

A. Data from all experimental combinations for the sex-linked recessive lethal mutation tests.

	Control	DMSO	DMSO Spermine	DMSO EMS	DMSO EMS Spermine	EMS
Total F_1 matings	400	351	776	293	302	271
Chromosome tested	367	329	718	270	289	201
Number of non-tests in F_1 matings	33	22	518	21	31	70
Number of F_2 scored normal	366	329	716	262	278	126
Number of F_2 scored lethal	1	0	2	7	11	73
Percent lethals	0.27%	0.0%	0.28%	2.50%	3.80%	36.2%

B. Chi-square value associated with comparison of the various mutation rates

	Control	DMSO	DMSO Spermine	DMSO EMS	DMSO EMS Spermine	EMS
Control	-	0.06	0.35	4.95	9.36	140
DMSO	0.06	-	0.04	6.48	9.73	-
DMSO-spermine	0.35	0.04	-	9.13	17.45	-
DMSO-EMS	4.95*	6.48**	9.13**	-	0.95	92.42
DMSO-EMS-spermine	9.36**	9.73**	17.45**	0.95	-	-
EMS	140.0**	-	-	92.42**	-	-

* significant at the 5% level with one degree of freedom
** significant at the 1% level or higher with one degree of freedom

mutation rate, shows a 17.45 chi-square value (Table 1). This chi-square value indicates that when EMS and spermine are applied together they are inducing a significant number of sex-linked recessive lethal mutation over the rate of spermine alone.

When the control group is compared to the DMSO-EMS-spermine treated flies, which demonstrate a 3.8% sex-linked recessive lethal mutation rate, a chi-square value of 9.36 is obtained (Table 1). This chi-square value indicates that DMSO-EMS-spermine treated flies have a mutation rate that is significantly greater than the control group at the 1% probability level. From this result one might speculate that spermine in the presence of DMSO and EMS is intereacting in such a manner that spermine might have a synergistic effect with EMS. However, when the mutation rates of DMSO-EMS-spermine treated flies are compared to the DMSO-EMS treated flies a chi-square value of 0.95 is obtained (Table 1). This chi-square value indicates that the mutation rates between DMSO-EMS-spermine and DMSO-EMS are not significantly different, at the 5% probability level. This low chi-square value between DMSO-EMS-spermine and DMSO-EMS suggests that spermine is not acting synergistically with EMS.

A 36.2% sex-linked recessive lethal mutation rate was obtained on flies that were treated by feeding 0.025M EMS in 1.0% sucrose suspended on absorbent paper. When this mutation rate is compared to the control group a 140.0 chi-square

value results (Table 1). This high chi-square value indicates the EMS used in this experiment was capable of producing a highly significant sex-linked recessive lethal mutation rate when administered orally.

When the sex-linked recessive lethal mutation rate of DMSO-treated flies is compared to the sex-linked recessive lethal mutation rate of DMSO-EMS, a chi-square value of 6.48 is obtained (Table 1). Such a high chi-square value indicates that EMS is producing a significant sex-linked recessive lethal mutation rate and since the mutation rate of DMSO treated flies is 0.0%, EMS is solely responsible for the 2.5% mutation rate unless there is a synergistic action between DMSO and EMS that can not be detected by the tests in this experiment (Table 1).

Comparing DMSO treated flies which have a 0.0% mutation rate and DMSO-spermine treated flies which have a 0.28% mutation rate, a chi-square of 0.04 is obtained (Table 1). This chi-square value strongly indicates that the addition of spermine is not acting as a mutagen in the presence of DMSO, and that the treatment of DMSO or DMSO-spermine have no significant effect upon the sex-linked recessive lethal mutation rate.

When DMSO treated flies are compared to DMSO-EMS-spermine treated flies a significant difference in sex-linked recessive lethal mutation rates is observed. The DMSO-EMS-spermine treated flies have a 3.8% sex-linked

recessive lethal mutation rate. The chi-square value between these two groups is 9.73 (Table 1).

Post Fertilization Mortality Tests

The results of the post fertilization mortality tests (Table 2) indicate the chemicals used had various effects upon the viability of the eggs. In the control group 825 eggs were counted out of which 749 eggs hatched, giving a 90.7% hatching rate.

The flies that received the DMSO-EMS-spermine treatment had a 95.0% hatching rate. The DMSO-EMS-spermine treated flies represents the only group that showed a higher hatching rate than the control group. When the control group and the DMSO-EMS-spermine group were compared, a chi-square value of 10.0 was calculated. This chi-square value indicates that the three chemicals apparently have depressed the hatching rate to a significant degree as compared with the non-treated controls such that the probability of this event would be less than 1% (Table 2).

All other tests showed a hatching rate that was below the hatching rate of the control group. All tests that did show hatching rates below the controlled group were compared to the control group. In each case the chi-square was 11 or above. This large chi-square value indicates that chemicals being tested have a highly significant effect upon hatching rates which is reflected in the increased post fertilization

Table 2. Post-fertilization mortality tests.

A. All data from all experimental combinations for post-fertilization mortality tests.

	Control	DMSO	DMSO Spermine	DMSO EMS	DMSO EMS Spermine	EMS
Number of eggs	825	1033	592	1027	813	813
Number of eggs non-hatched	76	493	118	141	42	102
Number of eggs hatched	749	540	474	886	771	256
Percent of normal egg development	90.7%	52.3%	68.5%	86.3%	95.0%	71.5%

B. Chi-square values associated with various combinations of post-fertilization mortality tests.

	Control	DMSO	DMSO Spermine	DMSO EMS	DMSO EMS Spermine	EMS
Control	-	320.0	34.9	11.0	10.0	71.9
DMSO	320.0**	-	18.2	289	405	-
DMSO-spermine	34.9**	18.2**	-	107.0	74.2	-
DMSO-EMS	11.0**	289.0**	107.0**	-	36.4	-
DMSO-EMS-spermine	10.0**	450.0**	74.2**	36.4**	-	-
EMS	71.9**	-	-	-	-	-

* significant at the 5% level with one degree of freedom
** significant at the 1% level or higher with one degree of freedom

mortality.

Flies that were treated with DMSO had the lowest hatching rate, 52.3%. When spermine was added to DMSO the hatching rate increased to 68.5%. When EMS was added to DMSO the hatching rate increased to 86.3% (Table 2).

Flies that received EMS by means of feeding on a solution of 1.0% sucrose and 0.025M EMS suspended on absorbent paper had a 71.5% hatching rate.

pH Values

Research by Johnson and Bach (1968) indicate that the amount of spermine available to stabilize DNA decreases with lower pH values because the affinity between spermine and t-RNA increases with decreasing pH values.

Spermine and DMSO had the lowest pH value, 2.25. DMSO has the highest pH value, 6.65. The pH of the DMSO-EMS-spermine solution was 2.80. When 0.025M EMS is mixed with 1.0% sucrose the pH value is 4.3. When EMS is mixed with DMSO the pH drops to 3.9.

1.0M DMSO and 0.02M spermine_____2.25 pH

1.0M DMSO, 0.02M spermine and 0.025M EMS_____2.80 pH

1.0M DMSO and 0.025M EMS_____3.90 pH

0.025M EMS and 1.0% Sucrose solution_____4.30 pH

1.0M DMSO_____6.65 pH

DISCUSSION

The major objective of this experiment was to determine if spermine could act as an antimutagen in sperm cells of Drosophila when these sperm cells were exposed to a known mutagen, EMS. Both of these chemicals had to be carried across the exoskeleton of the abdomen, associated membranes and tissues of the body wall, through the tissues of the testes and seminiferous tubules to eventually have contact with the sperm cells.

The action of spermine is disputed. Spermine could be considered a poly-cationic ion which would behave similarly to calcium and magnesium ions. Behaving as a cationic substance spermine would be attracted to numerous anionic species within the cell. Therefore the amount of spermine that would actually combine with DNA is uncertain. However, investigations by Johnson and Bach (1968) suggest that 3 to 17% of the spermine that enters a bacterial cell will combine and stabilize DNA. Some spermine may attach to RNA, but its bond strength is less than the spermine-DNA bond strength. Some spermine may join with ribosomes, in this case the release of newly synthesized proteins is inhibited.

That spermine can stabilize the degradation of DNA is supported by evidence that the presence of spermine increases the melting temperature of DNA. The stabilization of DNA is affected by pH. As the pH decreases, the ability of spermine

to stabilize DNA increases. Apparently, the acid environment causes the non-covalent bond between the amine group of spermine and the phosphate group of the DNA molecule to form a stronger bond than can be formed in a higher pH environment (Hirschman, Leng and Felsenfeld, 1967).

The actual bonding mechanism and position of spermine on DNA is uncertain. However, the general accepted idea is that the amine groups of spermine interact with the phosphate groups of DNA and that the spermine molecule bridges across the DNA molecule. The amines of spermine most likely attaches to the phosphates of the adenine and thymine nucleotides (Stevens, 1967).

Spermine appears to work as an antimutagenic agent in the presence of penicillin and streptomycin. Drug-sensitive strains of Staphylococcus aureus and Aerobacter aerogenes cells were saturated in 100 micrograms of spermine for forty minutes and then irradiated for 5 minutes with U.V. light. After irradiation treatment, the bacteria strains were plated on antibiotic containing media. The viable count of the spermine treated cultures was approximately 170 times less than with similar cultures from which spermine had been omitted (Sevag and Drabble, 1962). This work may be of particular importance to the present experiment since EMS can cause radiomimetic mutations.

It is also possible that the spermine molecule could be oxidized into products that would be toxic to the cells.

The enzyme which accomplishes this oxidation (amine oxidase) has been found only in beef plasma (Hirsch, 1953).

The action of EMS in the cells is also disputed. Evidence suggests that EMS has its greatest effect on post-miotic sperm cells, and with asexual reproducing cells the greatest effect will be during the G_2 stage (Biesele, 1963).

The mutations that result from a monofunctional alkylating agent like EMS are probably not due to cross linking mechanism that might interfere with the action of DNA, but are most likely due to an "apurinic gap" that may produce a transition of guanine-cytosine pair to an adenine-thymine pair. This transition of base pairs could produce a missense or nonsense mutation (Auerback, 1967).

It is thought that the alkylating action of EMS is at the N-7 position of guanine. The alkylation of guanine could cause it to erroneously combine with thymine instead of cytosine. This would result in a transition mutation. EMS could also cause guanine to leave the DNA molecule resulting in a depurination gap. This gap could result in a different nucleotide sequence or a shortened nucleotide sequence. In this latter case a missense or nonsense mutation could result (Lawley and Brooks, 1963).

Of the 367 chromosomes tested in the control group only one demonstrated a sex-linked recessive lethal mutation, resulting in a 0.27% recessive lethal mutation rate (Table 1). This sex-linked recessive lethal mutation rate is

slightly higher than the 0.0% sex-linked recessive lethal mutation rate observed by Alexander (1966), following a 15 day mating period. The chi-square value between the sex-linked recessive lethal mutation rate observed by Alexander and the sex-linked recessive lethal mutation rate observed in this test is 0.06. This low chi-square value indicates that the sex-linked recessive lethal mutation rate of the control group in the present experiment does not differ from that of the mutation rate observed by Alexander and represents a normal spontaneous mutation rate for this strain of Drosophila.

However, there is a difference in the egg hatching rate of the control group in Alexander's experiment and the present experiment. Alexander observed a 95.6% hatching rate using 2236 eggs in her test. The hatching rate in the present experiment was 90.7% using 825 eggs (Table 2). The chi-square value between these two hatching rates is 24.8. The larger chi-square value suggests a significant difference at the 5% level between these two observations. Possible explanation for this difference might be: (1) females were not fully inseminated, (2) refusal of the female to mate in the allotted period of time, (3) male sterility, (4) stock difference, and (5) treated males may lack the desire to mate.

The flies treated with DMSO had a sex-linked recessive lethal mutation rate of 0.0% (Table 1) which is not significantly different, at the 5% level, from the control value

for these experiments. Also, the sex-linked recessive lethal mutation rate of flies treated with DMSO in this experiment is identical to the sex-linked recessive lethal mutation rate observed by Alexander for her work with DMSO treated flies.

From these two similar observations it may be suggested that DMSO has no effect upon the sex-linked recessive lethal mutation rate. However, DMSO may have a significant effect upon the dominant lethal mutation rate as measured by means of egg hatching to determine post-fertilization mortality. The dominant lethal test in this experiment is quite different from that observed by Alexander. In the present experiment the egg hatching rate of DMSO treated flies was 52.3% (Table 2). Alexander recorded a 94.9% post-fertilization mortality rate for flies treated with DMSO.

The difference in post-fertilization mortality for flies with DMSO might be explained by one or all of the following: (1) The concentration of DMSO used in this experiment was ten-times greater than the concentration that Alexander used. Alexander used a 0.1M while 1.0M concentration was used in this experiment. (2) Alexander allowed the male and female to mate for three days while this experiment's mating period was one day. It is possible that the DMSO treatment partially inactivated the sperm or interfered with the males ability to copulate. (3) Alexander dipped the abdomens for a short period of time while the present

experiment placed the abdomen of the fly in a drop and left the fly's abdomen in the drop until the fly recovered from the anesthesia or until the drop evaporated. The method used in this experiment would permit more DMSO to pass into the abdomen and more would eventually reach the testes.

Flies that fed for twenty-four hours upon a mixture of 1.0% sucrose and 0.025M EMS suspended upon absorbent paper, produced a 36.2% sex-linked recessive lethal mutation rate (Table 1). Khan (1969) obtained similar results by feeding Drosophila on 0.012M EMS with a similar feeding method for twenty-four hours. Khan recorded a 43.6% sex-linked recessive lethal mutation rate from eggs layed within three days after mating. He also obtained a 38.7% sex-linked recessive lethal mutation rate for eggs that were stored in females for six days after having mated with the treated males. In this last experiment of Khan's, females were kept on a food medium consisting of 4% agar and 2% glucose (Khan, 1969). The high mutation rate obtained in this experiment indicates that the EMS used was capable of producing a significant number of mutations when ingested.

When 0.025M EMS was carried into the testes by DMSO, the sex-linked recessive lethal mutation rate dripped to 2.59% (Table 1). A 4.95 chi-square value (Table 1) between DMSO and DMSO-EMS indicates that EMS did penetrate the abdominal wall and did cause a significant increase, at the 5% level, in the sex-linked recessive lethal mutation rate.

Since the mutation rate of DMSO treated flies was 0.0%, one might conclude that the agent responsible for the increased mutation rate was EMS.

When the sex-linked recessive lethal mutation rate of flies fed on 0.025M EMS and 1.0% sucrose is compared to the sex-linked recessive lethal mutation rate of flies treated with DMSO-EMS, the mutation rate drops from 36.8% to 2.59%. This large drop in mutation rate may indicate that the combination of DMSO with EMS causes a reduction in the effectiveness of EMS. Or, that the amount of EMS that actually entered the seminiferous tubules was much less than the original amount applied. DMSO had to carry EMS through the exoskeleton, abdominal membrane and various tissues before reaching the final destination, the sperm cells. It is possible that DMSO lost some or most of its molecular carrying ability on its path to the sperm cells in the seminiferous tubules.

The amount of EMS that would enter the sperm cells would be difficult to determine. Epler's (1966) investigation indicated that the rate of sex-linked recessive lethal mutations is related to the number of EMS molecules that might reach the sperm cells. Epler was able to produce a 15.83% sex-linked recessive lethal mutation rate by injecting 5 microliters of 0.02M EMS into the abdomens of Drosophila. Using the same quantity, but increasing the molar concentration to 0.03M, Epler was able to increase the sex-linked

recessive lethal mutation rate to 19.56% (Epler, 1966). Since the mutation rate of the present experiment was 2.59% for 5 microliters of a 0.025M solution applied topically, it might be assumed that the quantity of EMS that entered the abdomens was less than 5 microliters.

Much of the EMS may have become attached to anionic agents that are in the path to the sperm cells, or some of the EMS may have been diluted and disseminated throughout the body via the circulatory system. Feeding EMS to Drosophila involves similar problems. The amount of EMS any one fly ingested may have varied from fly to fly. At any rate, the amount of EMS that actually combines with DNA in the sperm and is actively functioning to produce mutations may not always be the same amount from one fly to the next regardless of the method of treatment.

The combination of DMSO and spermine produced a sex-linked recessive lethal mutation rate of 0.28% (Table 1). This sex-linked recessive lethal mutation rate was quite similar to the sex-linked recessive lethal mutation rate of the control group which was 0.27% (Table 1). The DMSO-spermine treatment produced a sex-linked recessive lethal mutation rate that was slightly higher than the DMSO mutation rate, but it was not significantly higher. The chi-square value being 0.04 (Table 1). This low chi-square value suggests that DMSO and spermine entering the abdominal region will not cause a significant increase in the sex-linked

recessive lethal mutation rate.

Investigation by Johnson and Bach (1968) on the uptake of tritiated spermine by intact cells of Escherichia coli and spheroplasts have shown that spermine will have an increasing affinity for t-RNA at pH values of 4.0 and below. The pH value of DMSO-spermine solution was 2.25. At this pH it is possible that a large amount of the spermine could combine with t-RNA. Also, it is possible that the highly charged spermine molecule may have become attached to anionic agents of the cell membranes.

The hatching rate of DMSO treated flies was 52.3% (Table 2). This hatching rate is considerably different from the 94.9% hatching rate that Alexander (1966) observed for DMSO treated flies. However, the DMSO applied in this experiment was ten times greater in concentration than was used by Alexander. This higher concentration of DMSO may have affected the copulating abilities of the flies treated. Flies that received the higher concentrations of DMSO may have been partially sterilized.

The mutation rate of DMSO-EMS treated flies was 2.59% (Table 1). When spermine was added to DMSO and EMS, a 3.8% rate was observed (Table 1). The increase in the sex-linked recessive lethal mutation rate appears to be due to a synergistic effect of spermine, but when the sex-linked recessive lethal mutation rates of DMSO-EMS and DMSO-EMS-spermine treated flies are compared, a 0.95 chi-square value is

(Table 1). This chi-square value indicates that there is no significant difference in the mutation rates of flies treated with DMSO-EMS and DMSO-EMS-spermine. A 0.95 chi-square value would have an associated probability between 0.90 and 0.70 for one degree of freedom.

If spermine could act as an antimutagen in higher organisms, such as insects; it was hoped that the tests between DMSO-EMS and DMSO-EMS-spermine would indicate this activity. However, the results indicate that spermine may not have any significant effect upon the sex-linked recessive lethal mutation rate under the present test conditions. The slight enhancement of the mutation rate with the addition of spermine to DMSO and EMS is contradictory to the hypothesis stated in the introduction. It is possible that the spermine in the DMSO-EMS-spermine solution had a greater affinity for the anionic agents than did the EMS. This could possibly free more EMS molecules to attack the DNA molecule, thus producing more mutations.

When the sex-linked recessive lethal mutation rates on DMSO-spermine treated flies and DMSO-EMS-spermine treated flies are compared, an increase from 0.28% to 3.80% is observed. The chi-square value between these two groups is 17.45 (Table 1). This highly significant chi-square value indicates that the addition of EMS has a strong and significant influence on the induction of sex-linked recessive lethal mutations. The increased mutation rate of DMSO-EMS-

spermine above the DMSO-spermine mutation rate indicates that EMS can function to produce mutations when spermine is present. Apparently, there is little or no chemical interaction between EMS and spermine as measured by these tests.

The DMSO-EMS-spermine treated flies had the best egg hatching percentage (95.0%) of all the treated groups including the control group (Table 2). It is possible that the combination of DMSO, EMS and spermine would neutralize the affect that any one individual drug might have upon the dominant lethal mutation rate as observed by means of egg hatching tests. The 9.3% dominant lethal mutation rate (Table 2) that occurred for the control group in the present experiment is high when compared to the 4.4% dominant lethal mutation rate of Alexander (1966), but the high rate observed in this experiment may represent a variation that sometimes occur with Drosophila. However, when the hatching rate of the DMSO-EMS-spermine (95.0%) treated flies is compared to the hatching rate of the control group of Alexander's experiment (95.6%) one can observe very little difference.

Johnson and Bach (1965) suggest that spermine might act as an antimutagen in the presence of an antibiotic such as penicillin or streptomycin. The development of resistant cells to penicillin and streptomycin occurs by means of a selection process of a new mutation by the antibiotic. The antibiotic does not induce the mutation but merely allows the mutation to manifest itself by showing a resistance to

the normal action of the antibiotic. The development of resistant cells to penicillin is a result of individual cells to form an enzyme (penicillinase) that is not normally produced by the bacteria strain in question. The ability of a bacteria to produce penicillinase is a result of a spontaneous mutation. Bacteria cells that become resistant to streptomycin do so at the site of the ribosome. Streptomycin causes coding failures between m-RNA and t-RNA at the ribosome. The mechanism for cells to become resistant to streptomycin is not completely understood, but the mechanism is not due to a mutation of the DNA (Davis, Dulbecco, Eisen, Ginsberg and Wood, 1970).

Is it possible that spermine can stabilize the DNA in the presence of an antibiotic like streptomycin or penicillin better than it can in the presence of a strong mutagen like EMS? Also, is it possible that spermine has a greater attraction to the active site on the ribosome than does streptomycin? If so, spermine may be acting as an inhibitor to the normal function of streptomycin on the ribosome (Tabor and Tabor, 1964).

The major objective of this experiment was to determine if spermine could act as an antimutagen in Drosophila melanogaster when carried across the abdomen by DMSO. Since the sex-linked recessive lethal mutation rate of DMSO and DMSO-spermine treated flies is not significantly different from the sex-linked recessive lethal mutation rate of the

control group, DMSO and the combination of DMSO and spermine does not significantly affect the sex-linked recessive lethal mutation rate.

When DMSO is combined with EMS and applied to the abdomen a significant sex-linked mutation rate occurs when compared to the mutation rate of the control group. Another significant difference is observed when the DMSO-EMS treated flies are compared to the flies that fed upon a sucrose-EMS solution. The significant drop in sex-linked recessive lethal mutation rate of DMSO-EMS treated flies from the mutation rate of sucrose-EMS fed flies indicates that only a fraction of the applied EMS in the DMSO-EMS solution became effective in producing mutations in the sperm.

Flies that were treated with DMSO-EMS-spermine showed no significant difference in sex-linked recessive lethal mutation rate from the DMSO-EMS treated flies. Therefore, it appears that spermine does not act as an antimutagen in Drosophila melanogaster.

Post-fertilization mortality tests indicate that the concentration of DMSO may have affected the dominant lethal mutation rate or may have had an affect on the copulating abilities of the treated males. When DMSO is mixed with spermine or EMS or both, the affect of DMSO upon the hatching rate becomes less severe. Apparently, both EMS and spermine neutralize the drug action that DMSO has upon the flies. Since DMSO produced no sex-linked recessive lethal mutations,

it would be difficult to consider DMSO acting as a mutagen only on the autosomes. Therefore the affect that DMSO has on the hatching rate is probably one of a drug action affecting the copulating or fertilizing ability of the treated male flies.

CONCLUSION

The major objective of this experiment was to determine if spermine could act as an antimutagen in an organism that is more complex than bacteria. Earlier investigations by Johnson and Bach (1965) indicate that spermine can act as an antimutagen in bacteria.

The results of the present experiments indicate that spermine does not manifest any antimutagenic properties when applied with the mutagen, EMS. The sex-linked recessive lethal mutation rate of flies topically treated with 0.02M spermine and 0.025M EMS and 1.0M DMSO was slightly higher, but not significantly higher than the sex-linked recessive lethal mutation rate of flies that received a 1.0M DMSO and 0.025M EMS solution applied topically.

Topical application of 1.0M DMSO and 0.02M spermine does not have a significant effect upon the sex-linked recessive lethal mutation rate when compared to the control group and DMSO treated flies.

Flies that were fed on a solution of 1.0% sucrose and 0.025M EMS produced a 36.2% sex-linked recessive lethal

mutation rate. This mutation rate is similar to other sex-linked recessive lethal mutation rates that have been produced in Drosophila melanogaster by feeding of a similar concentration.

Flies that received DMSO treatments produced a 0.0% sex-linked recessive lethal mutation rate. This mutation rate is identical to the sex-linked recessive lethal mutation rate that was obtained by Alexander (1966).

However, the DMSO treatment did produce a 52.3% hatching rate. It is difficult to suggest that the low hatching rate observed with DMSO treatment is a result of dominant lethal mutations, since DMSO treatment did not cause any mutation on the X-chromosomes as observed by the sex-linked lethals test.

When spermine is added to DMSO the hatching rate increases to 86.3%. When EMS is added to the DMSO-spermine solution the hatching rate changes to 95.0%. The change in hatching rate may be due to a neutralization effect of DMSO by spermine and EMS.

Flies that were fed on 0.025M EMS in a 1.0% sucrose solution produced a 71.5% hatching rate. This low hatching rate indicates that the concentration of EMS used in this experiment was capable of producing a significant number of dominant lethal mutations when ingested.

LITERATURE CITED

Alderson, Thomas. 1965. Chemically induced delayed germinal mutation in drosophila. Nature 207:164-167.

Alexander, Mary. 1966. Dimethyl sulfoxide treatment of Drosophila, p. 505-516. In: Studies in Genetics III. Morgan centennial issue. Marshal R. Wheeler (Ed.), University of Texas Press, Publication number 6615.

Ashwood-Smith, M. J. 1961. The radio protective action of dimethyl sulfoxide and various other sulfoxides. Int. J. Radiat. Biol. 3:41-48.

Auerbach, C. 1967. The chemical production of mutations. Science 158:1141-1147.

Bautz, E., and E. Freeze. 1960. On the mutagenic effect of alkylating agents. Proc. Nat. Acad. Sci. 46:1585-1592.

Berry, S. J., and W. Dietz. 1968. The action of dimethyl sulfoxide and DNase on the fine structure of chironomid salivary gland cells. J. Insect Physio. 14:847-854.

Biesele, John J. 1963. Some morphological effects of alkylating agents. Exp. Cell Res. (Suppl. 9):525-534.

Davis, Bernard D., Renato Dulbecco, Herman N. Eisen, Harold S. Ginsberg, and W. Barry Wood. 1970. Microbiology. Harper and Row, New York. 316-323.

Dixon, Robert L., R. Adamson, M. Ben, and D. Rall. 1965. Apparent lack of interaction between dimethyl sulfoxide (DMSO) and a variety of drugs. Proc. Soc. Exp. Biol. Med. 118:756-759.

Epler, J. L. 1966. Ethyl methanesulfonate-induced lethals in Drosophila-frequency-dose relations and multiple mosaicism. Genetics 54:31-36.

Franz, T. J., and J. T. Van Bruggen. 1967. A possible mechanism of action of DMSO. Anu. N. Y. Acad. Sci. 141:302-307.

Hirsch, J. G. 1953. Spermine oxidase: An amine oxidase with specificity for spermine and spermidine. J. Exp. Med. 97:345-355.

Hirschman, Shalom Z., M. Leng, and Gary Felsenfeld. 1967. Interaction of spermine and DNA. Biopolymers 5:227-233.

Johnson, H. G., and M. K. Bach. 1965. Apparent suppression of mutation rates in bacteria by spermine. Nature 208:408-409.

Johnson, Herbert G., and Michael K. Bach. 1968. Uptake and subcellular localization of tritiated spermine in Escherichia coli. Arch. Riochem. Biophys. 128:113-123.

Khan, A. H. 1969. Effect of storage of alkylated chromosomes on the mutagenic effectiveness of monofunctional alkylation. Mutation Res. 8:565-571.

Lawley, P. D., and R. Brookes. 1963. The action of alkylating agents on deoxyribonucleic acid in relation to biological effects of the alkylating agents. Exp. Cell Res. (Suppl. 9):512-520.

Lindsley, Dan L., and E. H. Grell. 1967. Genetic Variations of Drosophila melanogaster. Carnegie Institute of Washington. Pub. 627. p. 22-268.

Lovelock, J., and M. W. H. Bishop. 1959. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature 183:1394-1395.

Parker, A. J. 1962. The effects of solvation on the properties of anions in dipolar aprotic solvents. Quarterly Rev., London: Chem. Soc. 16:163-169.

Sevag, M. G., and W. T. Drabble. 1962. Prevention of the emergence of drug-resistant bacteria by polyamines. Biochem. Biophys. Res. Commun. 8(6):446-452.

Spencer, Warren P., and Curt Stern. 1948. Experiments to test the validity of the linear r-dose/mutation frequency relation in drosophila at low dosage. Genetics 33:43-74.

Stevens, L. 1967. Studies on the interaction of homologues of spermine with deoxyribonucleic acid and with bacterial protoplasts. Biochem. J. 103(3):811-815.

Tabor, Herbert, and Celia W. Tabor. 1964. Spermidine, spermine, and related amines. Pharm. Rev. 16:245-287.